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Induction of Myogenesis in Mesenchymal Cells by MyoD Depends on Their Degree of Differentiation

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Expression of a transfected MyoD gene induces myogenic differentiation of most cell types. In this study, we evaluated the ability of an exogenous MyoD gene to induce myogenic conversion in two pairs of matched cell lines with different degrees of differentiation within either the osteoblastic or chondrocytic lineage. We show that osteoblasts and chondrocytes are resistant to the myogenic effects of MyoD alone. However, in their less-differentiated cell line counterparts, MyoD induces expression of muscle-cell-specific markers. Less-differentiated osteoblasts can be made resistant to MyoD-induced myogenic conversion by induction of adipogenic differentiation using dexamethasone. Finally, a dominant positive form of MyoD, one which is tethered to a partner, E47, activates muscle-specific gene expression in osteoblasts. Our results suggest that the response of a cell to MyoD depends on its lineage and its degree of differentiation. Furthermore, commitment of cells to the osteoblastic or chondrocytic lineage may involve inhibition of alternative pathways, such as those leading to myoblastic differentiation. Finally, osteoblasts may express a protein(s) which interferes with the activity of MyoD by inhibiting its association with E proteins. This interference can be overcome by expression of the MyoD–E47 hybrid, suggesting that osteoblasts are otherwise competent to undergo myogenic conversion.

INTRODUCTION

During development, the differentiation potential of cells becomes gradually restricted as they become committed to a defined lineage. Skeletal muscle cells are an excellent system to study the process of commitment, since their differentiation program involves distinct, well-characterized morphological and biochemical changes. Early studies of heterokaryons showed that muscle cells express proteins capable of activating muscle-specific genes in other cell types (Blau *et al.*, 1983). Subsequent studies led to the identification of the genes for MyoD (Davis *et al.*, 1987) and several related, muscle-specific transcription factors (reviewed in Edmondson and Olson, 1993) which can induce myogenic differentiation *in vitro* (reviewed in Olson, 1990; and Weintraub *et al.*, 1991) and *in vivo* (Hopwood *et al.*, 1991; Miner *et al.*, 1992). In addition, members of this family of basic helix–loop–helix (bHLH) proteins can activate their own, as well as each other's, expression in transfected cells, which may be important for the maintenance of the myogenic phenotype (Braun *et al.*, 1989; Thayer *et al.*, 1989; Edmondson *et al.*, 1991).

Forced expression of MyoD can induce expression of muscle-specific proteins in cells derived from many different lineages (Davis *et al.*, 1987; Weintraub *et al.*, 1989; Choi *et al.*, 1990; Boukamp *et al.*, 1992). For this reason, MyoD has been called a ''master regulatory gene'' for myogenesis (Weintraub *et al.*, 1989). MyoD activates transcription of muscle-specific genes by associating with a member of a family of ubiquitous bHLH proteins, called E proteins (Murre *et al.*, 1989a,b; reviewed in Kadesch, 1992), and binding to defined sequences, called E boxes, upstream from these genes (Ephrussi *et al.*, 1985; Church *et al.*, 1985). The fact that MyoD can convert most cell types to a myogenic phenotype suggests that no other tissue-specific proteins are required for MyoD activity (Weintraub *et al.*, 1989). Rather, MyoD activity can be regulated by ubiquitous proteins, some of which may also function in growth control or in other differentiation processes. For example, expression of the ubiquitous transcription factor pRB, which plays a key role in regulation of the cell cycle, is required for MyoD activity (Gu *et al.*, 1993). Furthermore, some tumors or tumor-derived cell lines, which exhibit uncontrolled growth and lose expression of their intrinsic differentiation program, are resistant to MyoD-induced differentiation (Weintraub *et al.*, 1989; Dias *et al.*, 1992; Tapscott *et al.*, 1993). Finally, other tissue-specific bHLH proteins, such as SCL/TAL and MASH, also heterodimerize with the same E pro-

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teins as MyoD, suggesting that the ubiquitous E proteins are involved in hematopoietic and neural cell differentiation (Hsu *et al.*, 1991; Johnson *et al.*, 1992) as well as in myogenic differentiation.

For cells of mesenchymal origin, expression of a myogenic phenotype is correlated with a loss of expression of the intrinsic differentiation program and vice versa. For example, adipocyte cell lines transfected with MyoD yield colonies of either muscle or fat cells, but not cells expressing both fat and muscle markers (Weintraub *et al.*, 1989). Similarly, myoblasts treated with thiazolidinediones or fatty acids (Teboul *et al.*, 1995) or transfected with adipogenic transcription factors (Hu *et al.*, 1995) lose their ability to differentiate into myotubes and gain the ability to differentiate into mature adipocytes. In addition, treatment of myoblasts (Katagiri *et al.*, 1994) or pluripotential cells (Yamaguchi *et al.*, 1991) with bone morphogenetic protein-2 (BMP-2) induces expression of osteoblastic markers and inhibits myogenic differentiation. Finally, expression of MyoD in chondroblasts leads to loss of expression of cartilage-specific markers (Choi *et al.*, 1990). This apparent mutual exclusivity of myogenic differentiation with adipogenic, osteoblastic, or chondrocytic differentiation suggests that these processes may compete for a limited amount of defined cellular protein(s). In contrast, in cells from other, nonmesenchymal origins, such as epithelial (Boukamp *et al.*, 1992) and neural crest cells (Weintraub *et al.*, 1989), the myogenic phenotype can exist in conjunction with the endogenous differentiation program. Thus, the differentiation response of cells to MyoD may depend on their lineage ''history'' (Schafer *et al.*, 1990). Consequently, cells which are close in lineage to muscle, such as those of mesenchymal origin, may require a mechanism(s) which leads to mutual exclusivity of differentiation programs and protects against myogenic conversion in case of aberrant MyoD expression. In contrast, more distantly related cell types, such as epithelial cells, might not have such a mechanism. Furthermore, the ability of a cell to undergo MyoD-induced myogenic conversion might be used as a diagnostic measure to evaluate whether a mesenchymal cell is committed to a particular lineage, and may provide insight into the mechanistic basis of such commitment.

To further understand the process of commitment, we tested whether expression of MyoD has the ability to induce myogenic differentiation of well-characterized mesenchymal cell lines. In addition to adipogenic or myogenic phenotypes, mesenchymal stem cells can be induced to express proteins found in osteoblastic or chondrocytic cells (Taylor and Jones, 1979; Yamaguchi and Kahn, 1991; Grigoriadis *et al.*, 1988, 1990). Although the sequence of expression of some of these markers is not yet known, specific proteins, such as osteocalcin, found in osteoblasts within bone, and collagen X, found in chondrocytes within hypertrophic cartilage, are considered to be markers of more mature cells. Other proteins, such as alkaline phosphatase and collagen II, are expressed in areas of bone and cartilage, respectively, which contain less mature cells. Based on these *in vivo* observations, as well as on the temporal expression pattern of markers in primary cultures *in vitro,* certain cell lines may be ''more mature'' than others due to their expression of specific proteins. For our studies, we have used two pairs of closely related cell lines, one pair to represent osteoblastic cells and the other as characteristic of chondrocytic cells. Both cell lines within each pair were derived at the same time from the same tissue and differentially express specific markers. As such, these cell lines may represent different stages of progression along their respective pathways, i.e., osteoblastic or chondrogenic. For this reason, we refer to cells which express little, if any, of the early differentiation markers and none of the later ones as ''less differentiated.'' Those cells which express high levels of early markers, and may express later ones as well, are considered to be ''more differentiated.'' Our studies show that, in both the osteoblastic and chondrocytic lineages, only the less-differentiated cells can undergo myogenic differentiation after expression of an exogenous MyoD gene. These results suggest that the ability of a cell to respond to MyoD is determined by its intrinsic differentiation state. However, the more differentiated osteoblastic cells could be induced to express a myogenic phenotype by transfection with a ''dominant positive'' version of MyoD, in which MyoD is tethered to E47 (Neuhold and Wold, 1993), suggesting that these cells are otherwise competent to undergo myogenic conversion. Thus, our data support the hypothesis that, for cells which are close in lineage to myoblasts, the ability to undergo MyoD-induced myogenesis is lost as cells become more differentiated along an alternative pathway.

MATERIALS AND METHODS

Cell Culture and Transfections and Infections

ROB C20 and C26 cells, originally purified by limited dilution subcloning of cells migrating from explants of parietal bones of 1-day-old rats (Yamaguchi and Kahn, 1991), were obtained from Dr. A. Kahn and grown in 10% fetal calf serum (FCS) in DMEα without nucleosides. RCJ3.1 C12 and C5.18, which were subcloned by limiting dilution of cells from sequential collagenase digestion of 21-day fetal rat calvariae (Grigoriadis *et al.*, 1988, 1990), were obtained from Dr. J. Aubin and grown in 15% fetal calf serum (FCS) in DMEα with nucleosides, in the presence or absence of dexamethasone (10^{-7} *M*). C2C12 cells (Yaffe and Saxel, 1977; Blau *et al.*, 1983) were obtained from Dr. Helen Blau and were grown in 20% FCS in DMEM. To induce myogenic differentiation, parental and transfected or infected cells were switched into differentiation media consisting of DMEM with 2% horse serum or DMEM with insulin, transferrin, and selenium (GMS-G, Gibco, BRL) for 3 to 5 days.

Using the calcium phosphate precipitation method (Sambrook, 1989), C20 cells were stably transfected with MyoD (Davis *et al.*, 1987) subcloned into an expression vector containing the CMV promoter and encoding neomycin resistance (pcDNAINeo, In-Vitrogen). In subsequent experiments, C20 cells were stably co-transfected with plasmids encoding neomycin resistance and with vectors containing the SV40 promoter driving expression of MyoD alone, E47 alone, or the MyoD–E47-tethered protein (Neuhold and

Wold, 1993). The transfected cells were then cultured in medium containing G418 (400 μg/ml). After 14 to 21 days, G418-resistant clones were isolated and expanded, and the MyoD-transfected cells were screened by Northern hybridization for expression of mRNA for MyoD. Colonies of the C20 cells transfected with the MyoD–E47 construct were immediately subcloned and screened for myogenic conversion since expression of this construct would be expected to inhibit growth (Sorrentino *et al.*, 1990) and in other cell lines, stable clones could not be obtained (Neuhold and Wold, 1993).

For retroviral infections, we used a high-titer MyoD retrovirus which was kindly supplied by Dr. Dusty Miller (Weintraub *et al.*, 1989). Similar results were obtained following infection with a lower titer virus encoding MyoD and hygromycin resistance (Lassar *et al.*, 1989a). Viral infections were performed by incubating growing cells overnight with viral stock plus 4 mg/ml of polybrene. Two days after infection, cells were switched to medium containing either G418 (400 μg/ml) for the high-titer virus or hygromycin (500 μg/ml) for the lower-titer virus, to select for infected cells.

Treatment of C26 cells with dexamethasone (10^{-7} *M*) and assessment of the resultant adipocyte phenotype by oil red O staining was performed as previously described for these cells (Yamaguchi and Kahn, 1991).

Northern Hybridization Analysis

Cells were cultured in growth media (20% fetal calf serum) or were switched to differentiation media for 3–5 days. RNA was isolated (Chomczynski and Sacchi, 1987) and analyzed by Northern blotting (Sambrook, 1989) using cDNA probes radiolabelled by random priming (Boehringer Mannheim). Following hybridization at 42°C for 24 hr, the nylon filters (Genescreen, NEN research products) were washed in 0.5× SSC, 0.5% SDS for 20 min at 42°C, and 0.1× SSC, 0.5% SDS, twice for 30 min at 55°C. cDNAs for myosin light chain 2, MyoD (Davis *et al.*, 1987), Id-1 (Benezra *et al.*, 1990), Id2 (Sun *et al.*, 1991), Id3 (Christy *et al.*, 1991), and myogenin (Wright *et al.*, 1989) were used as hybridization probes to assess induction of myogenic differentiation.

Western Analysis

Equal quantities of protein (determined by the Bio-Rad colorimetric assay) were analyzed by electrophoresis in 12% (for troponin T, myogenin, or MyoD) or 7.5% (for pRB or myosin heavy chain) denaturing polyacrylamide gels and transferred to nitrocellulose membranes at 40 V for 2 hr. The blots were washed for 10 min in TBST (25 m*M* Tris, pH 8.0, 125 m*M* NaCl, 0.025% Tween 20), incubated in blocking buffer (25 m*M* Tris, pH 8.0, 125 m*M* NaCl, 0.1% Tween 20, 1% BSA, 0.1% NaN_3) for 2 hr at room temperature or 24 hr at 4°C, and then incubated with the antibody for 2 hr at room temperature. They were then washed three times with blocking buffer (15 min per wash) and incubated with a secondary, alkaline phosphatase-conjugated antibody (Promega) for 1 hr. After three 15-min washes with blocking buffer and three 5-min washes with TBST, the membranes were incubated in phosphatase substrate (BCIP/NBT, Kirkegaard and Perry Laboratories) for 5–30 min and, once developed, washed with distilled water. Antibodies for troponin T were provided by Dr. Charles Ordahl (University of California, San Francisco), and those for myosin heavy chain (i.e., MF20 antibody) were purchased from the Developmental Studies Hybridoma Bank at NICHD. The antibodies to pRB (DeCaprio *et al.*, 1988) were purchased from Pharmingen. MyoD antibodies were kindly provided by Dr. Peter Dias and Dr. Peter Houghton (Dias *et al.*, 1992), and those for myogenin were received from Dr. Woodring Wright (Southwestern Medical School, Dallas, TX).

Immunofluorescence

Cells grown on coverslips were washed three times with phosphate-buffered saline (PBS) and fixed in methanol (for myosin heavy chain or collagen II antibodies) or in 4% formaldehyde in PBS (for MyoD) for 10 min at room temperature. After washing with PBS, cells were incubated in 0.1% Triton in PBS for 10 min and rinsed again with PBS. The samples were then treated with 3% bovine serum albumin (BSA) in PBS for 15–30 min, and incubated with primary antibody for 45 min at 37°C. After three washes in PBS, the cells were incubated with secondary antibodies (Jackson ImmunoResearch, West Grove, PA) which were either rhodamine-conjugated (for myosin heavy chain and MyoD antibodies) or fluorescein-conjugated (for collagen II antibodies) for 45 min at 37°C. The cells were then washed three times with PBS, mounted with fluorophore and a coverslip, and photographed with a Zeiss Axioplan microscope.

RESULTS

Expression of MyoD in Preosteoblasts and Osteoblasts

For our transfection studies, we used two cell lines, ROB C20 and C26, which were concomitantly derived from the same rat calvarial cell preparation (Yamaguchi and Kahn, 1991). Previous studies have shown that these cells differentially express osteoblastic markers, and thus may represent different stages of osteoblastic differentiation. C20 cells express several features of the osteoblastic phenotype: high levels of the osteoblastic form of alkaline phosphatase, cAMP production in response to parathyroid hormone and prostaglandin E2, and type I collagen synthesis (Yamaguchi and Kahn, 1991). Because C20 cells express only this phenotype and no other, C20 cells are considered to represent osteoblasts. In contrast, C26 cells express a much lower level of alkaline phosphatase and are capable of expressing alternate phenotypes. For example, C26, but not C20 cells, can differentiate into adipocytes by treatment with dexamethasone (Yamaguchi and Kahn, 1991). Because C26 cells express low levels of some osteoblastic markers and can be induced to form a mineralized matrix, C26 cells are considered to represent less-differentiated cells, i.e., ''preosteoblasts.''

C20 cells were transfected with an expression plasmid for the myogenic transcription factor MyoD under the control of the CMV promoter, and stable clones were established. Among the approximately 50 G418-resistant clones, only one expressed MyoD mRNA. This clone did not express mRNA for myosin light chain 2 or myogenin, two

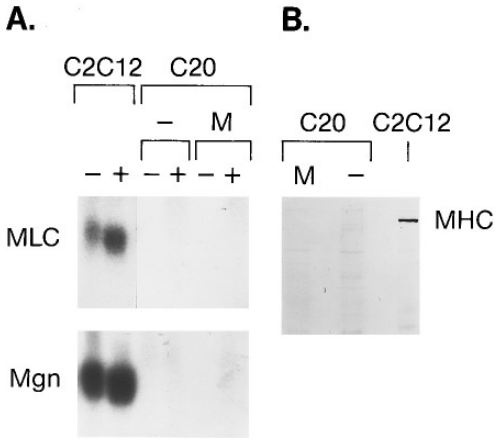

FIG. 1. Expression of muscle-specific mRNAs in MyoD-expressing C20 osteoblasts. (A) Northern blot analysis of mRNA for myosin light chain (MLC) or myogenin (Mgn). RNA was prepared from untransfected (−) or transfected, MyoD-expressing (M) C20 cells, cultured in growth (−) or differentiation (+) medium for 3 days. RNA from C2C12 myoblasts, cultured under the same conditions, was included as a positive control. Equal amounts of RNA (5 μg) were analyzed. The myosin light chain and myogenin expression in C2C12 myoblasts in growth medium (−) is due to spontaneous differentiation of a fraction of confluent cells, as previously described (Lassar *et al.,* 1989; Filvaroff *et al.,* 1993). (B) Western blot analysis using antibodies to myosin heavy chain (MHC). Equal quantities of protein from untransfected (−) or MyoD-expressing (M) C20 osteoblasts grown under differentiation conditions were analyzed. Differentiated C2C12 myoblasts were included as positive control.

markers of muscle cell differentiation (Fig. 1), nor did these cells fuse to form myotubes when cultured in differentiation medium, i.e., low serum which normally induces myoblasts to form multinuclear myotubes (data not shown). Because MyoD expression is known to inhibit cell growth (Sorrentino *et al.,* 1990; Crescenzi *et al.,* 1990), MyoD-expressing cells are likely to be lost during selection of stable clones, which may explain the low incidence of MyoD-expressing stable clones.

We therefore infected cells with a recombinant retrovirus that directs MyoD expression. We used a retroviral vector with a high multiplicity of infection so that most cells would contain the exogenous MyoD gene and analysis of these cells could be done within 1 week of infection. After infection, C20 and C26 cells expressed MyoD mRNA (data not shown) and MyoD protein (Fig. 2). These cells did not express endogenous MyoD mRNA as assessed by Northern analysis (data not shown), even though forced expression of a transfected MyoD gene activates endogenous MyoD expression in various cell lines (Lassar *et al.,* 1989a; Thayer *et al.,* 1989; Weintraub *et al.,* 1989). In differentiation medium, C26 cells infected with the MyoD retrovirus, C26Myo, but not the corresponding C20 cells, C20Myo, formed elongated, primarily mononucleated myofibers (Fig. 3). Accordingly, C26Myo, but not C20Myo, expressed myosin heavy chain and myogenin (Fig. 4). Uninfected C26 and C20 cells did not form myofibers (Fig. 3) nor did they express muscle marker proteins under similar conditions (Fig. 4). The MyoD protein in these nondifferentiating C20Myo cells was functional because it could cooperate with E47 (introduced by transient transfection) to activate transcription of an exogenous myosin light chain 1 enhancer/promoter–chloramphenicol acetyltransferase (CAT) construct (pMLC1CAT920; Rosenthal *et al.,* 1989) (data not shown; see below). Our results show that two closely related cell lines, C20 and C26, derived from the same passage of parental cells, display different abilities to be converted to the myogenic phenotype by forced expression of MyoD.

Dexamethasone Abolishes the Myogenic Phenotype of MyoD-Expressing C26 Cells

Our results suggest that during maturation of multipotential mesenchymal cells to fully differentiated osteoblasts, cells undergo a change(s) which confers resistance to the myogenic effects of MyoD. To further test the hypothesis that differentiation along one pathway precludes progression along another, we examined whether induction of C26 cells to an alternate phenotype would block their ability to express a myogenic phenotype. Because C26 cells differentiate into adipocytes in the presence of dexamethasone (Yamaguchi and Kahn, 1991), both parental C26 and C26Myo cells were treated with dexamethasone and tested for expression of myogenic differentiation markers. As previously reported (Yamaguchi and Kahn, 1991) and confirmed by oil red O staining of fat droplets (data not shown), dexamethasone induced the formation of adipocytes in both C26 and

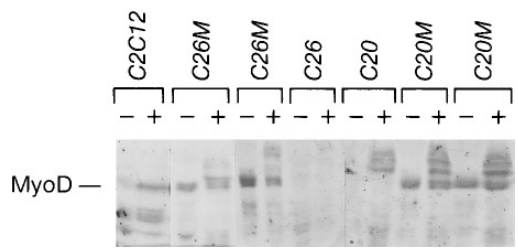

FIG. 2. Expression of MyoD protein in MyoD-expressing cells. Protein extracts were prepared from parental C20 osteoblasts or C26 preosteoblasts, or from cells infected with a MyoD-encoding retrovirus (C20M and C26M), cultured in growth (−) or differentiation (+) media. Each lane contains equal amounts of protein, which were analyzed together by Western blotting using MyoD antibody. C2C12 myoblasts cultured under the same conditions were included as a positive control. The two sets of lanes for C26M and C20M correspond to two different pools of retrovirally infected cells. In C20 and C20M cells, the antibody-reactive bands of higher molecular weight than MyoD that are induced by differentiation medium are very unlikely to represent MyoD variants because C20 cells do not express MyoD mRNA.

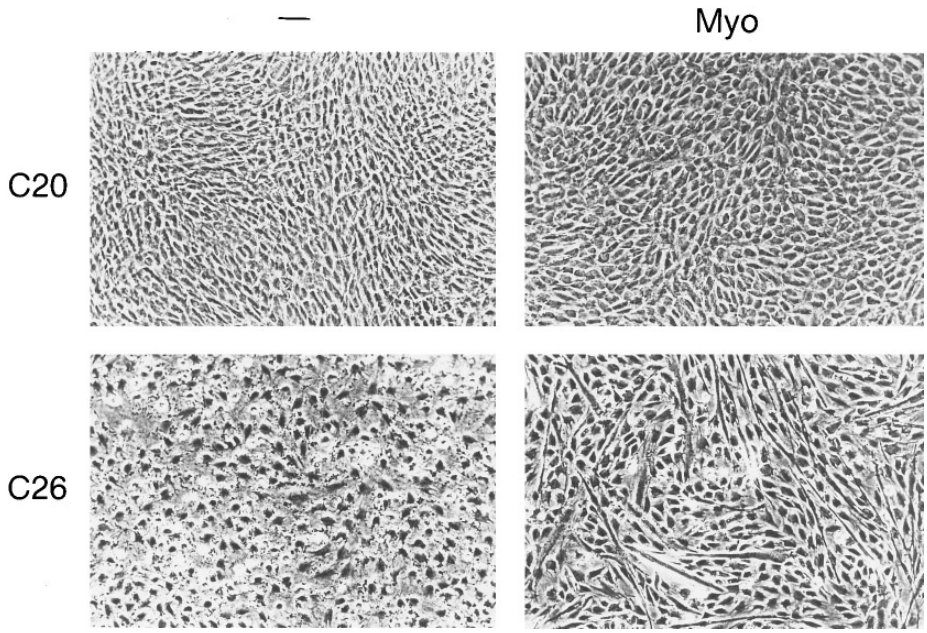

FIG. 3. Morphology of parental (−) and MyoD (Myo)-expressing C26 preosteoblasts and C20 osteoblasts. Cells were cultured in differentiation medium for 3 days and then photographed by phase-contrast microscopy to document myotube formation.

C26Myo cells. Concurrently, dexamethasone inhibited the ability of C26Myo cells to form elongated, predominantly mononucleated, myofibers (Fig. 5A) and to express myosin heavy chain and troponin T (Fig. 5B). Thus, induction of an adipocytic phenotype is associated with turning off expression of muscle-specific genes, even in cells with a retrovirally expressed MyoD gene. In agreement with previous findings (Weintraub *et al.,* 1989; Hu *et al.,* 1995; Teboul *et al.,* 1995), we conclude that the adipocyte and myoblast phenotypes are mutually exclusive.

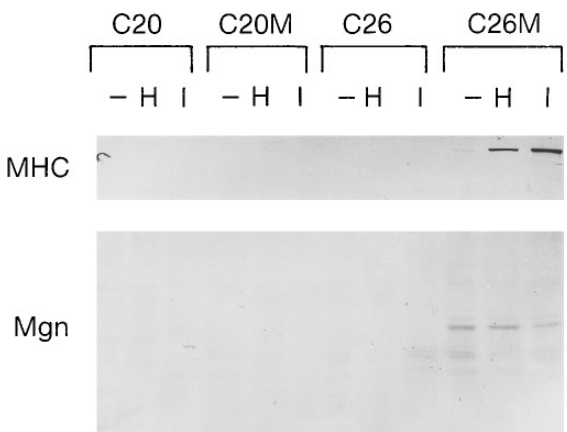

FIG. 4. Expression of muscle-specific proteins in parental or MyoD-expressing (M) C26 and C20 cells. Equal amounts of protein extracts of cells cultured in growth (−) or differentiation medium with 2% horse serum (H) or insulin (I) were analyzed by Western blotting using antibodies for myosin heavy chain (MHC) or myogenin (Mgn).

Expression of pRB, Id, and MyoD in C20 Cells

The inability of C20Myo cells to exhibit a myogenic phenotype could be due to the absence of some factor required for myoblast differentiation. For example, the osteosarcoma-derived cell line, Saos-2, is unable to undergo MyoD-induced myogenic differentiation due to expression of a truncated, nonfunctional form of the retinoblastoma gene product, pRB (Gu *et al.,* 1993). We therefore tested whether C20 cells expressed pRB protein. In a manner similar to normal cycling myoblasts, C20 cells in growth media (10% serum) expressed pRB in both its phosphorylated (upper band) and hypophosphorylated (lower band) forms (Fig. 6A, lane 2). Furthermore, C20 and C20Myo cells in differentiation media (2% horse serum) expressed pRB in its hypophosphorylated form (Fig. 6A, right), which is known to bind to MyoD (Gu *et al.,* 1993).

The inability of C20Myo cells to undergo myogenic differentiation could also be attributed to a high expression level of any one of the Id genes (Benezra *et al.,* 1990; Christy *et al.,* 1991; Sun *et al.,* 1991). By direct binding to E proteins, Id proteins prevent the association of E proteins with MyoD and the transcriptional activity of MyoD, which results in an inhibition of differentiation (Benezra *et al.,* 1990). As

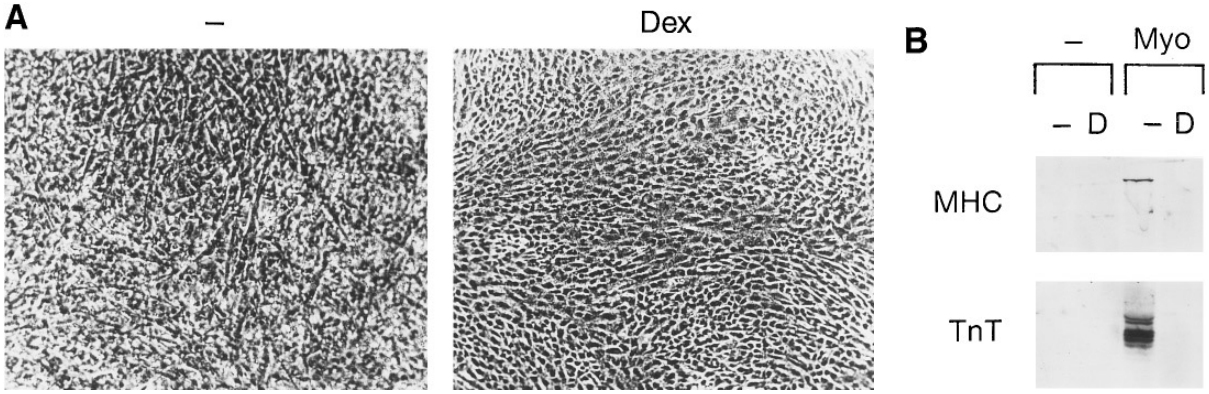

FIG. 5. Phenotype of MyoD-expressing C26 preosteoblasts treated with dexamethasone. (A) Morphology of C26 cells cultured in the absence (–) or presence of dexamethasone (Dex) for 9 days. Photography was carried out by phase-contrast microscopy. (B) Expression of muscle-specific proteins. Protein extracts prepared from parental (–) or MyoD-expressing (Myo) cells, untreated (–) or cultured with dexamethasone (D) for 9 days in growth medium, were analyzed by Western blot analysis for myosin heavy chain (MHC) or troponin T (TnT) expression.

shown in Fig. 6B (left), C20 cells expressed Id1 mRNA at a level which was slightly higher than that of C26 cells, but considerably lower than in the myoblast cell line C2C12, or in 10T1/2 cells, which can undergo MyoD-induced myogenic differentiation (Davis *et al.*, 1987). In addition, the Id1 levels in C20 cells in differentiation medium are comparable to or less than those in C2C12 myoblast cultures under the same conditions (Fig. 6B, right) in which C2C12 cells undergo myogenic differentiation (Blau *et al.*, 1983; Lassar *et al.*, 1989a). Id2 and Id3 mRNA levels were similar in C20 and C26 cells and were not significantly affected by expression of MyoD in these two cell lines (Fig. 6C). Finally, localization of MyoD protein in the cytoplasm rather than the nucleus is an additional way to inhibit MyoD-induced myogenesis (Rupp *et al.*, 1994). Therefore, we confirmed by immunofluorescence that the MyoD protein was present in the nucleus of C20 cells (data not shown). Thus, neither the absence of pRB nor high levels of Id1, 2, or 3 expression nor mislocalization of MyoD can explain the resistance of C20 cells to MyoD-induced myogenic conversion.

Expression of a Tethered MyoD–E47 Fusion Protein Induces Myogenic Conversion

The inability of C20Myo cells to differentiate could be due either to the presence of an inhibitor of MyoD activity or to the absence of a positive factor required for MyoD activity. To distinguish between these two possibilities, we utilized a MyoD–E47 hybrid protein, in which MyoD is tethered to a transcriptional partner E47 (Neuhold and Wold, 1993). Because this hybrid protein can activate muscle-specific genes even in the presence of inhibitory proteins, the MyoD–E47 protein is considered to act as a dominant positive form of MyoD (Neuhold and Wold, 1993). Expression plasmids for MyoD, E47, or the MyoD–E47 fusion protein were transfected into C20 cells, and stable clones were tested for their ability to undergo myogenic differentiation. C20 cells transfected with MyoD–E47, but not those transfected with either MyoD or E47 alone (data not shown), were able to form elongated fibers which expressed myosin heavy chain protein as detected by both immunofluorescence (Fig. 7A) and by Western analysis (Fig. 7B). Within the population of C20 cells transfected with MyoD alone, a few rounded, mononuclear cells occasionally stained weakly positive for myosin heavy chain (MHC) by immunofluorescence. However, the incidence of MHC-positive cells was considerably lower than that found in C20 cells transfected with tethered MyoD–E47 as reflected by Western analysis of this protein (Fig. 7B). In addition, transient transfections of C20 cells with MyoD and E47, either as separate proteins (*trans*) or as the tethered hybrid (*cis*), led to transcriptional activation of a muscle-specific promoter assessed using a chloramphenicol acetyltransferase reporter assay (Rosenthal *et al.*, 1989) (data not shown). Thus, in the presence of high levels of E proteins (in *cis* or in *trans*), MyoD was able to convert C20 cells to a myogenic phenotype, suggesting that these cells contain all factors necessary for induction of myogenesis by the MyoD–E47 complex.

MyoD Expression in Precartilage and Cartilage Cells

As shown above, the progression from a multipotential cell capable of becoming an osteoblast to a cell expressing several osteoblastic markers, as represented by the C26 and C20 cell lines, respectively, is accompanied by a loss of ability to be converted to a myogenic phenotype by MyoD. To test whether this phenomenon occurs during differentiation of other types of mesenchymal cells, we used another

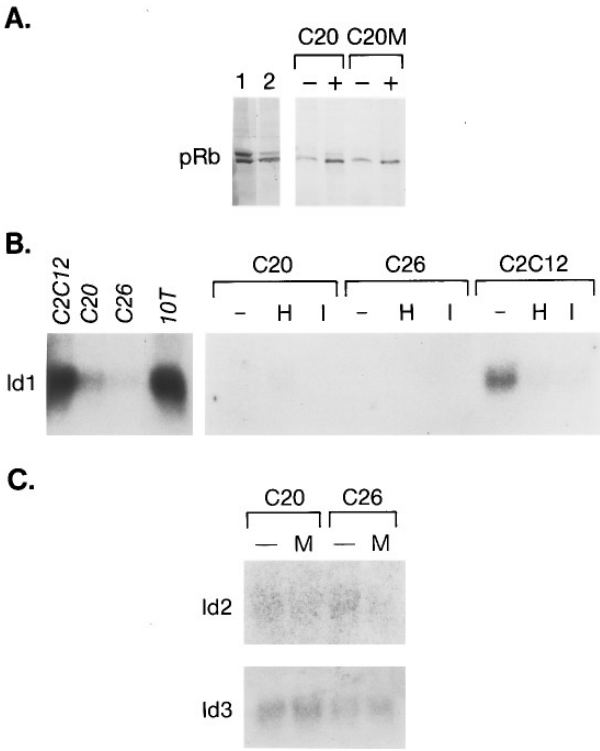

FIG. 6. Expression of the retinoblastoma protein pRB and Id mRNA in parental and MyoD-expressing C20 and C26 cells. (A) Detection of pRB by Western blot analysis of protein extracts prepared from proliferating C2C12 myoblasts (1) or C20 osteoblasts (2). In addition, protein extracts were prepared from parental or MyoD-expressing (M) C20 cells, cultured in growth (−) or differentiation (+) medium for 3 days. To allow myotube formation, cells were grown to maximal confluence, which may explain the relative abundance of hypophosphorylated pRB (the fastest moving pRB band) in cells in growth medium. (B) Detection of Id. mRNA by Northern hybridization in C2C12 myoblasts, C20 osteoblasts, C26 preosteoblasts, and 10T1/2 fibroblasts (10T) cultured in growth medium (left). In a separate experiment (right), RNA was prepared from C20 osteoblasts, C26 preosteoblasts, and C2C12 myoblasts cultured in growth medium (−) or differentiation medium with 2% horse serum (H) or insulin (I). Equal amounts (15 μg) of RNA were analyzed. (C) Detection of Id2 and Id3 mRNA by Northern hybridization in C20 osteoblasts (C20) or C26 cells (C26) parental (−) or the corresponding cells expressing MyoD (M).

matched pair of cell lines, the subclones C5.18 and C12, derived from the multipotential cell line RCJ3.1 (Grigoriadis *et al.*, 1988, 1990). C5.18 cells differentiate into chondrocytes spontaneously when cultured at high plating density, or in the presence of dexamethasone at lower density. In contrast, C12 cells do not differentiate into chondrocytes either spontaneously or in the presence of dexamethasone, but can be induced to form cartilage nodules by BMP-2 (50 ng/ml) at low plating density (Aubin *et al.*, personal communication). For this reason, C12 cells will be referred to as ''prechondrocytes.'' Because C5.18 cells are unipotential and can express collagen X, which is specifically found in hypertrophic cartilage, this cell line is likely to represent a more mature chondrocyte. Thus, similarly to the osteoblastic C26 and C20 cells, the C12 and C5.18 cells may represent two closely related stages of chondrocytic maturation, with C12 cells being less mature than C5.18 cells.

The C12 and C5.18 cells were infected with the MyoD-expressing retrovirus and were evaluated for their ability to express a myogenic phenotype. Infected C12Myo and C5.18Myo cells expressed MyoD protein as assessed by Western blotting (Fig. 8A). However, only C12Myo cells, but not C5.18Myo cells, formed spindle-shaped, predominantly mononuclear, fibers (Fig. 8B) and expressed myosin heavy chain, myogenin (Fig. 9) and troponin T (data not shown) when cultured in differentiation medium. Thus, the ability of MyoD to induce myogenesis in C12 cells, but not in C5.18 cells, correlates with their degree of differentiation within the chondrocytic lineage. These results are similar to those seen with the two ROB subclones, C26 and C20, where only the less-differentiated cells were converted to a myogenic phenotype by MyoD. Furthermore, as in C20 osteoblasts, transient transfection of C5.18 chondrocytes with the dominant positive form of MyoD (tethered to E47) activated transcription from the myosin light chain 1 promoter (data not shown), suggesting that RCJ5.18 have all factors necessary for activation of muscle-specific genes by the MyoD–E47 complex.

We then tested whether altering the differentiation state of RCJ3.1 cells with dexamethasone would affect their ability to be converted by MyoD. Dexamethasone induces

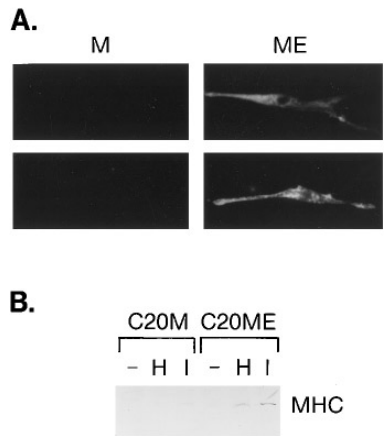

FIG. 7. Conversion of C20 osteoblasts to a myogenic phenotype by expression of the dominant positive MyoD-E47 protein. (A) C20 cells expressing either MyoD alone (M) or the MyoD-E47 fusion (ME) were stained by immunofluorescence using antibodies to myosin heavy chain. (B) Western blot detection of myosin heavy chain (MHC) in protein extracts of C20 cells expressing MyoD alone (M) or MyoD tethered to E47 (ME), cultured in growth medium (−) or differentiation medium containing 2% horse serum (H) or insulin (I) for 3 days.

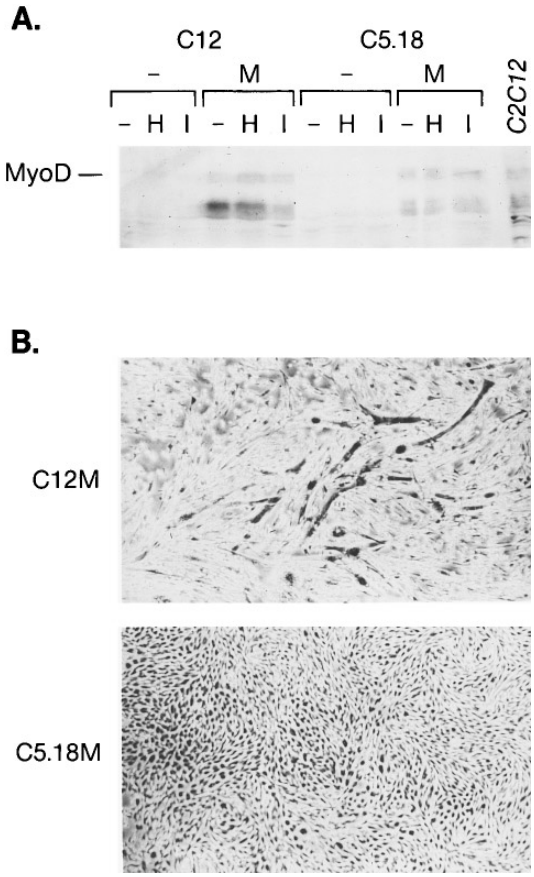

FIG. 8. Expression of MyoD protein in RCJ3.1 cells infected with a recombinant retrovirus, and its effect on morphology. (A) Western blot detection of MyoD in parental (−) or infected, MyoD-expressing (M) C12 prechondrocytes and C5.18 chondrocytes, cultured in growth medium (−) or differentiation medium containing 2% horse serum (H) or insulin (I). Equal amounts of protein extract were analyzed. The identity of the lower bands in the C12M and C5.18M cells is unknown, but they may represent degradation products. (B) C12 prechondrocytes (C12M) or C5.18 chondrocytes infected with a MyoD-encoding retrovirus were cultured in differentiation medium containing insulin for 3 days and photographed by phase-contrast microscopy.

chondrocytic differentiation of a number of pluripotential or prechondrocytic cell lines (Grigoriadis *et al.*, 1988, 1990). In the less-differentiated C12 cells, dexamethasone does not induce chondrocytic differentiation (Aubin *et al.*, personal communication) and did not affect the ability of C12Myo cells to express myoblast markers (Fig. 9). In contrast, dexamethasone induces chondrocytic differentiation of C5.18 cells (Aubin *et al.*, personal communication) as shown by expression of type II collagen, a cartilage-specific marker (Fig. 10). This induction did not occur in C5.18Myo cells (Fig. 10), suggesting that MyoD inhibited at least one aspect of the chondrocytic phenotype of these cells even though MyoD did not induce myogenic differentiation (Fig. 9). Surprisingly, treatment of C5.18Myo with dexamethasone and insulin, but not either agent alone, induced expression of myogenin (Fig. 9) and troponin T (data not shown) in these cells. Based on this result, we examined the effect of insulin and dexamethasone on the expression of the chondrocytic phenotype. Unlike dexamethasone alone, dexamethasone and insulin did not induce expression of type II collagen in C5.18 cells (Fig. 10). These results suggest that insulin inhibits dexamethasone induction of chondrocytic differentiation, which may thus facilitate myogenic differentiation of C5.18Myo cells. Our results support the idea that myogenic differentiation and other types of mesenchymal cell differentiation, such as chondrocytic, are mutually exclusive, and agree with previous findings that myogenic and chondrocytic markers are not expressed in the same cells (Choi *et al.*, 1990).

DISCUSSION

Our results show that less-differentiated osteoblasts and chondrocytes, but not their more mature counterparts, can be converted to a myogenic phenotype by expression of an exogenous MyoD gene. However, osteoblasts can be induced to express muscle-specific proteins by transfection with MyoD in its dominant positive form, i.e., tethered to E47 (MyoD–E47). The MyoD-induced conversion of the less-differentiated mesenchymal cells is consistent with previous observations that MyoD can induce expression of muscle-specific proteins in most, but not all, cell types (Davis *et al.*, 1987; Schafer *et al.*, 1990; Weintraub *et al.*, 1989; reviewed in Olson, 1990, and Weintraub *et al.*, 1991; Olson and Klein, 1994).

The inability of MyoD-expressing osteoblasts to undergo myogenic differentiation after introduction of an exogenous

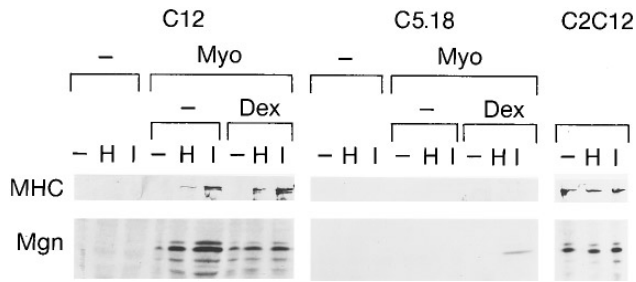

FIG. 9. Expression of myosin heavy chain and myogenin in C12 prechondrocytes and C5.18 chondrocytes. Parental (−) and MyoD-expressing (Myo) C12 and C5.18 cells were cultured in growth (−) or differentiation medium containing either 2% horse serum (H) or insulin (I) for 3 days, in the absence (−) or presence (Dex) of dexamethasone. Equal amounts of protein were analyzed by Western blotting using antibodies to myosin heavy chain (MHC) or myogenin (Mgn). C2C12 myoblasts cultured under similar conditions were included as a positive control.

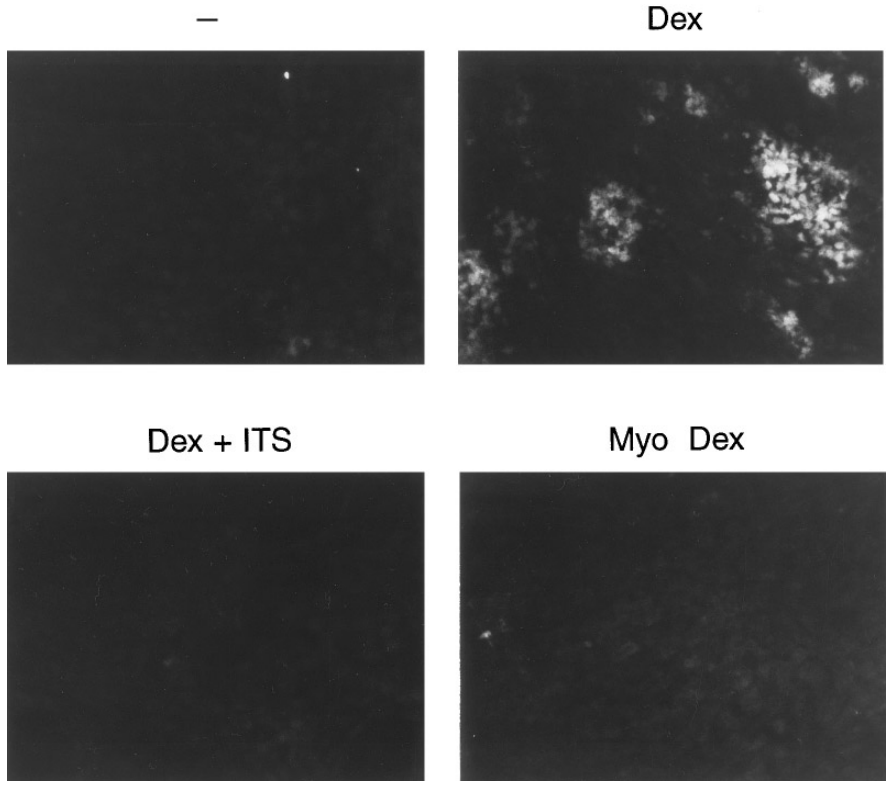

FIG. 10. Immunofluorescent detection of collagen II expression in C5.18 chondrocytes, cultured in growth medium (–) with dexamethasone (Dex) or dexamethasone and insulin (Dex + ITS), or in MyoD-expressing C5.18 cells treated with dexamethasone (Myo Dex).

MyoD gene is consistent with some studies on osteosarcoma cell lines. Saos-2 cells, but not U20S cells, are resistant to the effects of MyoD alone (Gu *et al.*, 1993). TE85 cells require both MyoD expression and treatment with the demethylating agent 5-azacytidine in order to undergo significant myogenic conversion (Chen and Jones, 1990). Thus, expression of genes other than MyoD must be altered in order to induce a myogenic phenotype in osteoblasts. In their study, myosin heavy chain-expressing cells continued to stain positive for alkaline phosphatase (Chen and Jones, 1990), a stable enzyme expressed in osteoblasts and other cell types (Nair *et al.*, 1987). The different responses of these osteosarcoma cell lines to MyoD may be related to their degree of differentiation because it has been suggested that tumor cells continue to express the phenotype apparent at the time of transformation (Greaves *et al.*, 1986). In contrast to our C20 cells, all osteosarcoma cell lines were derived from tumors for which the genetic lesion is unknown, and which may have uncoupled the processes of differentiation and proliferation. Therefore, these cells may not be truly representative of normal osteoblasts.

Our data also show that MyoD can convert prechondrocytes, but not chondrocytes to a myogenic phenotype. Previous studies have shown that introduction of the MyoD gene into primary chicken chondroblasts results in a low and variable degree of myogenic conversion and coincident loss of the chondrocytic phenotype (Choi *et al.*, 1990). However, in the control cultures, not all cells stained positive for early cartilage markers (Choi *et al.*, 1990), suggesting that this heterogeneous pool of cells may contain less-differentiated cells which are susceptible to conversion. These cell cultures may thus be comparable to our MyoD-sensitive RCJ3.1 C12 prechondrocyte line, rather than the MyoD-resistant RCJ3.1 C5.18 chondrocyte line which can express collagen X, a late marker of chondrocyte differentiation.

To evaluate the possible mechanistic basis of resistance of osteoblasts and chondrocytes to the differentiating effects of MyoD alone, one must consider how MyoD normally functions. To act as an efficient transcriptional activator of muscle-specific genes, the MyoD protein must associate with one of the ubiquitous E proteins, such as E47 or E12. The MyoD-E protein complexes bind to defined E box sequences upstream of muscle-specific genes and activate their transcription. The interaction between MyoD and E47 is required for several reasons. Not only does this heteromerization considerably enhance the DNA binding efficiency of MyoD compared to MyoD alone (Murre *et al.*, 1989b; Sun and Baltimore, 1991), but it also changes the DNA sequence binding specificity (Blackwell and Weintraub, 1990). Furthermore, association with E proteins alters

the phosphorylation state of MyoD and creates a more efficient transcriptional activator than the MyoD homodimer (Lassar *et al.*, 1991; Bengal *et al.*, 1994). MyoD activity can be regulated at each of these steps, and these individual events can be dissociated from each other. Accordingly, resistance to the myogenic activity of MyoD could be attributed to inhibition of any of these steps. However, in the case of C20 osteoblasts, a mechanistic basis for the resistance to myogenic conversion by MyoD is suggested by our finding that MyoD in combination with E47 separately (*in trans*) or tethered to MyoD (*in cis*) is able to induce myogenic differentiation. Indeed, these results suggest that an inability of endogenous E47 to associate with MyoD may be the limiting step in the myogenic conversion of C20 osteoblasts. In fact, transient transfection of C20Myo cells with an E47 expression plasmid results in activation of a muscle-specific promoter/enhancer–CAT construct. Furthermore, once the association of E47 and MyoD is favored by high levels of both proteins in *trans*, or in *cis* as a tethered MyoD–E47 fusion protein, C20 cells have all factors required for induction of myogenesis by the MyoD–E47 complex.

The resistance of C20 osteoblasts to myogenic conversion by MyoD alone and the dominant positive effect of MyoD and E47 in combination could in principle be attributed to low endogenous levels of E proteins. This possibility is unlikely since expression of E47/E12 is found in all cell lines tested (Murre *et al.*, 1989a; Zhuang *et al.*, 1992; Lenardo *et al.*, 1987), and is not altered during osteoblastic differentiation (Murray *et al.*, 1992). In addition, E47/E12 proteins are only two members of a family of E proteins which can bind to bHLH proteins and activate transcription, and may be functionally redundant in cultured cells (for review, see Kadesch, 1992). Moreover, antisense experiments suggest that E47/E12 expression is not essential for differentiation of embryonic stem cells into skeletal and cardiac muscle, erythrocytes, neurons, and cartilage (Zhuang *et al.*, 1992). Thus, in the absence of E47/E12, perhaps other members of the E protein family might functionally substitute.

A more likely explanation, however, for the requirement of a tethered MyoD-E47 protein to induce conversion of C20 cells, is that these cells express a factor which interferes with association of MyoD and E proteins. Based on the differential myogenic effect of MyoD on C26 and C20 cells, the expression or activity of such a factor is likely to be induced upon transition from multipotential cell to osteoblast. Several proteins have previously been shown to interfere with E47–MyoD association and subsequent myogenic conversion. The best studied inhibitors of myoblast differentiation are the Id proteins (Benezra *et al.*, 1990; Christy *et al.*, 1991; Sun *et al.*, 1991) which directly compete with MyoD for association with E proteins (Jen *et al.*, 1992). We have shown that expression of Id1, 2, and 3 is not significantly higher in osteoblasts than in their less-differentiated counterparts, which raises the possibility of another bHLH protein which binds to and complexes with E47 in C20 osteoblasts but not in the progenitor cells. By competing with MyoD for association with E proteins, such a bHLH protein could block the effect of MyoD alone but not that of either a tethered MyoD–E47 protein or high levels of both of these proteins in *trans*.

Several other lines of evidence suggest the involvement of bHLH proteins in osteoblast differentiation. First, levels of Id mRNA decrease during osteoblast differentiation (Ogata and Noda, 1991; Kawaguchi *et al.*, 1992; Murray *et al.*, 1992) and downregulation of Id is required for osteoblastic differentiation (Murray *et al.*, 1992). Second, many genes expressed in osteoblasts, including the osteoblast-specific osteocalcin gene, contain E-boxes to which bHLH proteins can bind (Siddhanti and Quarles, 1994). Finally, E-box binding activity increases during osteoblastic differentiation (Kawaguchi *et al.*, 1992). Thus, our results using the dominant positive MyoD further support the hypothesis that expression of a bHLH protein is induced during osteoblastic differentiation. Expression of such a protein could explain the apparent mutual exclusivity of myogenic and osteoblastic differentiation.

Whereas expression of a bHLH protein which competes with MyoD for E protein binding may best explain the inability of MyoD to convert C20 osteoblasts into myoblasts, we cannot exclude the possibility of other inhibitory mechanisms. Many proteins have been shown to affect MyoD activity. For example, c-jun and junB can associate with MyoD and thereby inhibit its activity (Bengal *et al.*, 1992; Li *et al.*, 1992), and high expression of c-myc (Miner and Wold, 1991) or c-fos (Lassar *et al.*, 1989a; Li *et al.*, 1992a) has been shown to inhibit myogenic differentiation. Inhibition of MyoD-induced myogenesis in osteoblasts could also be explained by expression of the homeobox proteins Msx1 and Msx2, which play key roles in skeletal bone development (Jabs *et al.*, 1993; Satokata and Maas, 1994) and can inhibit myogenesis (Song *et al.*, 1992; Woloshin *et al.*, 1995) by blocking expression of MyoD (Woloshin *et al.*, 1995). Expression of these proteins in C20 and C26 cells may explain the absence of autoinduction of the endogenous MyoD gene (Thayer *et al.*, 1989) observed in these cells (data not shown). Finally, MyoD is extensively phosphorylated (Tapscott *et al.*, 1988), and phosphorylation at specific sites can alter its activity (Li *et al.*, 1992b). Clearly the regulation of MyoD activity is complex, as MyoD can interact with many possible partners and can also be posttranslationally modified in multiple ways.

The study of cells resistant to the myogenic effects of MyoD has already proven useful in identifying proteins required for myogenesis (Gu *et al.*, 1993). Considering the mutual exclusivity of myogenic and adipocytic (Weintraub *et al.*, 1989, Hu *et al.*, 1995; Teboul *et al.*, 1995), osteoblastic (Yamaguchi *et al.*, 1991; Katagiri *et al.*, 1994), or chondrocytic (Choi *et al.*, 1990) phenotypes, the proteins involved in induction of adipocytic, osteoblastic, or chondrocytic differentiation may also be involved in resistance of more mature cells in these lineages to myogenic conversion. Thus, the study of two pairs of matched cell lines with differential sensitivities to the myogenic activity of MyoD, and repre-

senting different stages of osteoblastic or chondrocytic maturation, may help us to better understand the process of myogenic conversion. Comparative studies of these pairs of cell lines may also allow the identification of proteins—possibly bHLH proteins—which play a role in the commitment and differentiation of cells within the osteoblastic or chondrocytic lineage.

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